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Investigation of the Toxicologic and Biochemical Effects of Silk Fibroin/Gold Nanoparticles-Based Nanofiber Using Zebrafish Embryos

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ABSTRACT

Objective: This study aimed to test the toxicity of silk fibroin (SF) / gold nanoparticles (AuNPs)-based nanofiber by using zebrafish embryos as an alternative animal model.

Materials and Methods: Nanofiber was fabricated via electrospinning. The zebrafish embryos were divided into four groups as control, 3,4-dichloroaniline (DCA) treated, one day SF/AuNPs treated (1D), and seven days SF/AuNPs treated (7D) group. The SF/AuNPs nanofiber was incubated in the medium for one day and seven days. Following incubation, the embryos were placed in the mediums and their development was monitored 72 hours post-fertilization. In the zebrafish embryos, levels of malondialdehyde (MDA), nitric oxide (NO), activities of superoxide dismutase (SOD) and glutathione-S-transferase (GST) were detected.

Results: Compared to the control group, there was no change in the hatching and mortality rates in the 1D and 7D groups. In the DCA group, the mortality rate was higher than the controls. In the 1D and 7D groups, MDA and NO were higher than the control but lower than the DCA group. SOD and GST activities decreased compared to the control.

Conclusion: SF/AuNPs-based nanofiber did not affect the hatchability and mortality of embryos but increased oxidant damage, therefore it is thought that this oxidant effect of SF/AuNPs-based nanofibers may provide antibacterial properties.

Keywords: Silk fibrion, gold nanoparticles, zebrafish embryos, oxidant-antioxidant status, toxicity

INTRODUCTION

The development of functional nanofiber-based membranes has gained considerable interest due to their distinctive features like high level porosity, high surface area-to-volume ratio, and excellent biocompatibility (1). In this study, gold nanoparticles (AuNPs)-added silk fibroin (SF)-based nanofiber membranes were prepared. Among various materials studied for nanofibrous membranes, SF has attracted interest due to its biodegradability and low immunogenicity. SF is a natural protein produced by a variety of spiders and insect species that has remarkable strength, toughness, and flexibility (2). Human epithelial, fibroblast, keratinocyte, and osteoblast cells from a variety of tissues may adhere to, spread out, and develop when placed in three-dimensional SF nets. (3). SF has been utilized in bioengineering delivery systems for drugs, and delivery of therapeutics to cancer cells (4). SF has high biocompatibility, controlled degradability, structural integrity, and diverse process ability, these features make SF a desirable and valuable candidate for use in tissue science and delivery systems for drugs (5). Furthermore, SF nanofibers have been found to exhibit antioxidant effects, which can further support the wound-healing process

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(6). The addition of AuNPs to SF may exhibit bioactivities that could be used in biomedical applications. From a biological standpoint, the toxicity of AuNPs is crucial (7). In this case, the preparation of AuNPs, the dose and toxicity of AuNPs in products such as wound dressings in which nanoparticles are used needs to be considered. Following investigation of AuNPs in fibroblasts, epithelial cells, and macrophages, there was substantial concern that small AuNPs (dcore 2.0nm), which are more redox-reactive than bigger AuNPs, would have significant toxicity. However, further *in vitro* and *in vivo* studies have revealed that AuNPs, regardless of core diameter, are not acutely hazardous or toxic (8).

However, some studies state the AuNPs-induced reactive oxygen species production causes toxic effects on the DNA, cell nucleus, and mitochondria (9). Researchers have difficulty due to a lack of understanding of the mechanisms involved in the reduction stability and effects of biosynthesized gold nanoparticles (10). In this study gold nanoparticles were synthesized by using green chemistry techniques to decrease their hazardous effects and mixed with the SF to fabricate SF/AuNPs hybrid electrospun nanofiber.

The production of hybrid nanofibers containing gold nanoparticles are an important option in the invention of clean, nontoxic, and sustainable products with significant benefits over previous methods (11). Therefore, in this study, the possible toxicity of SF/AuNPs -based nanofibers was tested using zebrafish embryos focusing on the oxidant-antioxidant system when used as a wound dressing material.

MATERIALS AND METHODS

SF Preparation

SF was synthesized according to Zhu et al. (12). To degum the silk from sericin, Bombyx mori silk cocoons (5g) were cut into portions and boiled for thirty minutes in a 0.02 M sodium carbonate solution. The degummed silk was washed with deionized water and dried before being dissolved for four hours in 9.3 M LiBr solution and then dialyzed against deionized water for 72 hours to remove the LiBr. The obtained SF was centrifuged at 9,500xg and kept in 4°C.

AuNPs Preparation

AuNPs were prepared by using green chemistry. Collected fresh strawberry leaves were dried in a cool and shaded area. The dried strawberry leaves were then pulverized in a mortar. To ensure an equal size of the particles, the powder was sieved with a pore size of approximately 0.08mm sieve. 0.4 grams of strawberry leaf powder was mixed with deionized water and heated at 80°C for 20 minutes to create an aqueous extract. The plant extract was cooled to room temperature, and a simple centrifugation process was employed to remove the plant debris. A 1mM concentration of $HAuCl_4$ (chloroauric acid) solution was used to obtain gold nanoparticles. The 1mM $HAuCl_4$ solution was heated to 100^{0}C. The strawberry leaf extract was added to the boiling gold solution. Heating was continued until the prepared solution reached a dark red color. After the color change in approximately one to five seconds, the prepared solution was cooled to room temperature (13).

Preparation of SF /AuNP Nanofiber

10% SF in formic acid was mixed with 1% gold nanoparticles. This hybrid mixture was used to produce nanofiber. The optimized conditions for the electrospinning were determined as 25.5 kV electrical power, the flow rate in the feeding unit was 0.15mL per minute, the collector rotation speed was 200 rpm and the distance between the nozzle and the collector was 13.7 cm.

Development of Zebrafish Embryos

The zebrafish (AB/AB strain, wild type) was kept in an environment which was free of disease under controlled care. The zebrafish were maintained in an aquarium racking mechanism (ZebTEC, Italy) between 26-28^{0}C. They were fed with commercial powdered fish meal twice a day, mixed with live saltwater shrimp. All studies were conducted in 0.018 mg/L sea salt solution which was prepared with reverse osmosis water (Instant OceanTM, USA). For all experiments, reverse osmosis water supplemented with 0.018 mg/L sea salt (Instant OceanTM, USA) was used. After natural breeding, fertilized embryos were obtained, cultured, and classified based on development time and characteristic morphology using a Zeiss Discovery V8 stereomicroscope (Germany) (14).

Experimental Design of Zebrafish Embryo Groups

Zebrafish embryos were divided into four groups as control, 3,4-Dichloroaniline (DCA, Fluka, Steinheim, Germany), one-day (1D) and seven-day incubation (7D) groups. No treatment was applied to the embryo medium of the control group. In the DCA group, 4mg DCA/L was added to the embryo medium. In the 1D incubation group, 3x3cm SF/AuNPs based nanofiber was, continuously shaken in the embryo medium for 24 hours, and in the 7D incubation group 3x3 cm SF/AuNPs based nanofiber was continuously shaken in the embryo medium for seven days. Embryos were exposed to four different embryo mediums and monitored for 72 hours post-fertilization (hpf).

Biochemical Analysis

Preparation of Zebrafish Embryos for Biochemical Analysis

Biochemical analyses were conducted on the zebrafish embryos. The embryos were produced as five biological replicates of zebrafish embryos (100 embryos/pool) through the use of 72 hours post-fertilization (hpf) zebrafish. One milliliter phosphate-buffered saline (PBS) was used to homogenize 100 embryos in each pool and then the pools were centrifuged. Biochemical analysis was performed using the supernatant.

Lipid Peroxidation and Nitric Oxide (NO) Levels

The Ledwozyw method was used to measure the level of malondialdehyde (MDA), which serves as a lipid peroxidation end product, using thiobarbituric acid reactive substances (15). The MDA level was presented as nmol MDA/mL.

The NO measurement was conducted using the Miranda et al. method (16). In the NO determination, vanadium (III) chloride was used to reduce nitrate to nitrite. Under acidic conditions, the nitrite produced reacts with sulfonyl amide and N-(1-naphtyl) ethylenediamine dihydrochloride, forming a complex diazonium compound. The intensity of the resulting complex was measured at 540nm. The results were presented as µmol NO/mL.

Superoxide Dismutase (SOD) and Glutathione-S-Transferase (GST) Activities

The SOD activity was measured with a method that consist on the potential of SOD to enhance the effects of riboflavin-sensitized photooxidation of o-dianisidine. The resulting product's absorbance was determined at 460nm. The net absorbance was calculated by comparing the absorbance values at zero and eight minutes of illumination. The SOD activity was presented as U/mL (17).

The GST activity was measured using the Habig et al. method. The absorbance of the formed product was detected at 340nm wavelength using a spectrophotometer (18).

Statistical Analysis

One-way analysis of the variance (ANOVA) and post hoc Tukey's multiple comparison tests were used to analyze the differences between the groups using the Graph Pad 8 (GraphPad Software, La Jolla, CA, USA). $p<0.05$ was considered significant.

RESULTS

Development, Mortality and Hatching Rates of the Embryos

The mortality rates of zebrafish embryos at 72 hpf were presented in Figure 1. The mortality rate was significantly increased in embryos exposed to DCA when compared to the control group. Mortality rates in the 1D and 7D exposure groups were not significantly changed when compared to the control group. The hatching rates of zebrafish embryos at 72 hpf are presented in Figure 2. There was no significance between the hatching rates of all groups.

Lipid Peroxidation and NO Levels

The MDA and NO levels significantly increased in all groups when compared to the control group (Figure 3). The MDA and NO levels of the 1D and 7D groups were significantly lower than the DCA group (Figure 3).

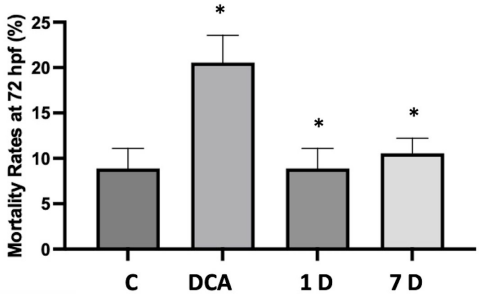

Figure 1. The mortality rates of zebrafish embryos exposed to DCA and also SF/AuNPs for 1 day (1D) and 7 days (7D) were measured at 72 hours post-fertilization (hpf).
C:Control
DCA: 3,4-Dichloroaniline
1D: Silk fibroin/gold nanoparticles treatment at day 1
7D: Silk fibroin/gold nanoparticles treatment at day 7.
*p< 0.05 significantly different from the control group (C).

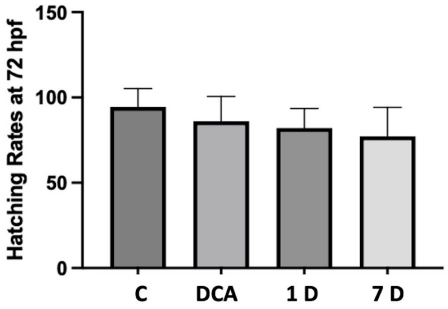

Figure 2. The hatching rates of zebrafish embryos exposed to DCA and also SF/AuNPs for 1 day (1D) and 7 days (7D) were measured at 72 hours post-fertilization (hpf).
C: Control
DCA: 3,4-Dichloroaniline
1D: Silk fibroin/gold nanoparticles treatment at day 1
7D: Silk fibroin/gold nanoparticles treatment at day 7.

SOD and GST Activities

The SOD activity in all groups was lower than the control group (Figure 4). The GST activity was significantly decreased in the DCA and 1D groups when compared to the control group, while the 7D group showed similar GST activity to the control group (Figure 4).

Discussion

In health-related products, gold nanoparticles and SF-based products continue to generate great attention. However, since there are dilemmas related to products containing these substances in the studies, assessing the potential toxicity

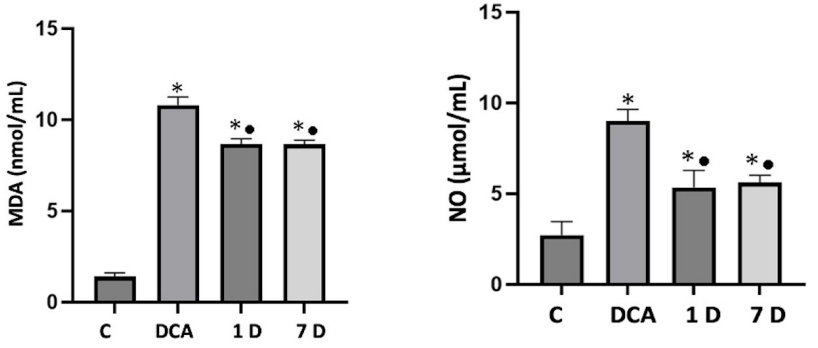

Figure 3. Malondialdehyde and nitric oxide levels of zebrafish embryos at 72 hpf.
C: Control
DCA: 3,4-Dichloroaniline
1D: Silk fibroin/gold nanoparticles treatment at day 1
7D: Silk fibroin/gold nanoparticles treatment at day 7,* $p < 0.05$ significantly different from the control group, • $p < 0.05$ significantly different from the DCA group

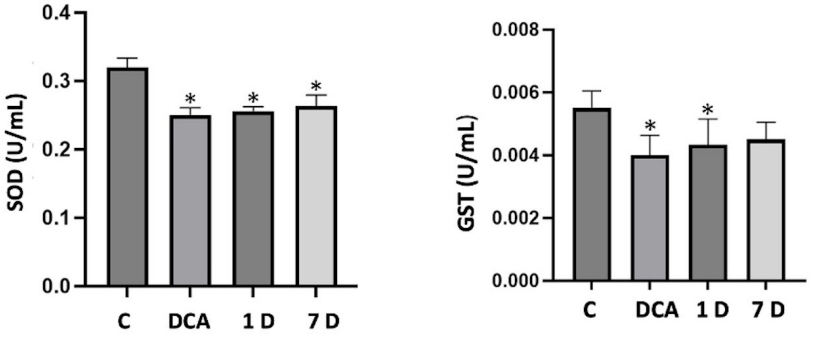

Figure 4. Superoxide dismutase and glutathione-S-transferase activities of zebrafish embryos at 72 hpf.
C: Control
DCA: 3,4-Dichloroaniline
1D: Silk fibroin/gold nanoparticles treatment at day 1
7D: Silk fibroin/gold nanoparticles treatment at day 7 * $p < 0.05$ significantly different from the control group.

of the SF/AuNPs-based hybrid nanofiber was necessary. Therefore, this study demonstrated the possible toxic and oxidant-antioxidant effects of SF/AuNPs-based nanofibrous membranes. According to the results of this study, the SF/AuNPs-based nanofibers had a nontoxic effect on zebrafish embryos and promoted the healthy development of zebrafish embryos compared to the DCA exposed zebrafish embryos as positive control. Higher hatching rates and lower mortality rates were observed in groups treated with SF/AuNPs -based nanofiber when compared to the DCA-exposed zebrafish embryos. It was revealed that gold nanoparticles do not exhibit any toxicity to zebrafish embryos when compared to other nanoparticles like silver, copper, and platinum (19). However, there is no research on how gold nanoparticles affect the zebrafish embryos when they form a hybrid with SF. The toxicity of SF/AuNPs hybrid nanofibers depend on several factors, including the characteristics of the nanoparticles, their concentration, exposure duration, and the specific biological system under consideration. While SF is generally considered biocompatible, the addition of gold nanoparticles could potentially introduce new properties and interactions that may affect the toxicity (20).

Exposure to SF/AuNPs-based nanofiber increased lipid peroxidation in the embryos. Under certain conditions, gold

nanoparticles may release and could contribute to oxidative damage. They could induce the generation of reactive oxygen. Excessive production of reactive oxygen species production disrupts the oxidant-antioxidant balance and leads to oxidative stress. The release of gold ions depends on factors such as pH, temperature, and exposure duration (21, 22). In this study, the increased lipid peroxidation level and decreased SOD and GST activities in the zebrafish embryos could be related to the gold nanoparticles released from the hybrid nanofiber. NO is another reactive oxygen species, and the current study found that embryonic NO levels increased in response to DCA and nanofibrous membranes (23). NO is a highly reactive physiological molecule that has been revealed to play a vital function in controlling pre-implantation embryo development (24). Gouge et al. revealed the need for NO in embryos before implantation and suggests that it may play a function in controlling mitosis in them (25). The present findings show that DCA and nanofibrous membrane resulted in higher NO generation in 72 hpf embryos when compared to the control group but this increase in the NO level did not affect the hatching and mortality rate of the zebrafish embryos. The oxidant effect of the SF/AuNPs nanofiber membrane exposed embryo medium showed that gold nanoparticles were released into the medium. This oxidant effect of the membrane could contribute to killing the bacteria by adhering to their surface and could cause oxidant damage to bacteria when used as a wound-healing material. This confirmed that the membrane produced in this study could have antibacterial properties. The dimension, shape, charge, and coating of the surface of gold nanoparticles released to the embryo medium could also influence their oxidant-antioxidant balance (26). The size of the gold nanoparticles that were used in this study was approximately 20 nm. Smaller nanoparticles, especially those below ten nanometers, have greater potential for cellular uptake and interaction and could alter the behavior of gold nanoparticles (27).

In conclusion, while SF is considered biocompatible, the addition of gold nanoparticles introduces a new component that could have an oxidant and therefore antibacterial effect on the hybrid nanofibers. Therefore, a careful evaluation of the specific hybrid nanofiber formulation is necessary to assess its safety and potential toxicity in the intended application.

Ethics Committee Approval: According to the legal regulations in the European Union and in our country, ethical committee approval is not required for the use of embryos and larvae in studies involving zebrafish during the first 120 hours of post-fertilization development. In this study, the research was terminated at 72 hours post-fertilization.

Authors' Contributions: Conception/Design of Study – O.O., T.T.A., E.E.A.; Data Acquisition – E.E.A., I.U., O.O., E.T.; Data Analysis/Interpretation – T.T.A., E.E.A.; Drafting Manuscript– T.T.A., O.O; Final Approval and Accountability– T.T.A., E.E.A.

Conflicts of Interest: The authors declare no conflict of interest.

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